Genetic polymorphism of glutathione S-transferase P1 (GSTP1) gene and its association with bladder cancer susceptibility

A dissertation submitted to The Dr. M.G.R. Medical University, Tamil Nadu, in partial fulfilment of the requirements for M.Ch. Branch-IV (Genitourinary surgery) examination to be held in August 2014

DEPARTMENT OF UROLOGY
CHRISTIAN MEDICAL COLLEGE, VELLORE

BONAFIDE CERTIFICATE

This is to certify that the work presented in this dissertation titled "**Genetic polymorphism of glutathione S-transferase P1 (GSTP1) gene and its association with bladder cancer susceptibility**" done towards fulfilment of the requirements of the **Tamil Nadu Dr. M.G.R. Medical University, Chennai for the M.Ch (Branch– IV) (Urology)** exams to be conducted in August 2014, is a bonafide work of the candidate **Dr. Vikas Rampal**, Senior Post graduate student in the Department of Urology, Christian Medical College, Vellore under my guidance and supervision. This dissertation has not been submitted, fully or in part to any other board or University.

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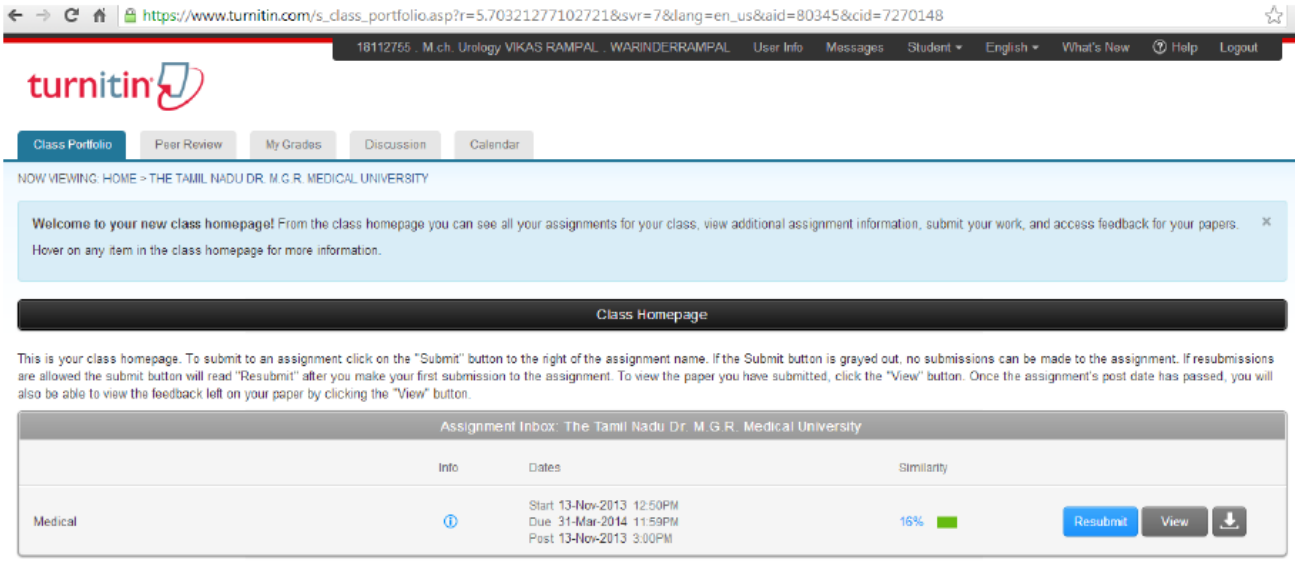
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Introduction

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Genetic polymorphism of glutathione S-transferase P1 (GSTP1) gene and its association with bladder cancer susceptibility in patients presenting to Christian Medical College and Hospital, Vellore.
Dr. Vikas Rampal, Senior Registrar, Urology, Dr. Nitin S Kekre, Urology, Dr. Sumita Danda, Medical Genetics, Dr. Arabind Panda, Urology.

Ref: IRB Min. No. 8063 dated 06.11.2012

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Dr. Nitin S Kekre, Department of Urology, CMC.
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*Last but not the least, I am proud to be a part of this prestigious institution, **Christian Medical College**, which has stood out as an important landmark in this fast rising metropolis and a ray of hope to the multitudes of destitute, poverty stricken people of this state and beyond, who arrive here daily from far, often as a last resort, to treat their illness.*

CONTENTS

ABBREVIATIONS

TCC	**Transitional Cell Carcinoma**
UC	**Urothelial Carcinoma**
GSTP1	**Glutathione S Transferase P1**
CIS	**Carcinoma in Situ**
PCR	**Polymerase Chain Reaction**
RFLP	**Restriction Fragment Length Polymorphism**
GWAS	**Genome Wide Association Studies**
Genotype AA	**Homozygous wild type Isoleucine- isoleucine**
Genotype AG	**Heterozygous isoleucine- valine**
Genotype GG	**Homozygous polymorphic valine- valine**

Introduction

Bladder cancer is one of the most common urological malignancies. As per the Indian cancer registry data, it is the ninth most common cancer in men accounting for 3.9% of all cancer cases (1). In the middle-aged and elderly men; bladder cancer is the second most prevalent malignancy after prostate cancer. It is well documented that the tobacco smoke and occupational carcinogens promote epithelial cell dysplasia and they are the most common environmental cause of urothelial carcinoma of the bladder. Most of these carcinogens are detoxified by phase II metabolic enzymes like Glutathione S transferase. It is hypothesized that polymorphisms in genes encoding these enzymes, affects susceptibility to develop urothelial cancers (UC). One of the extensively studied genes in this group is glutathione S-transferase P1 [GSTP1]. Studies of the relationship of polymorphisms of GSTP1 and UC of bladder have been equivocal, with some studies claiming positive associations with GSTP1 polymorphism while the others claiming a negative association. This present study aims to assess the association between genetic polymorphism of GSTP1 gene and development of UC of bladder in patients presenting to Christian Medical College and Hospital, Vellore.

Aims and objectives

Primary:

- **To detect any association of genetic polymorphism of glutathione S-transferase P1 (GSTP1) gene with transitional cell carcinoma of the bladder**

Secondary

- **To assess if the risk of transitional cell carcinoma of the bladder is increased in smokers with polymorphism of GSTP1 gene polymorphism**

Review of Literature

Epidemiology of bladder cancer:

Carcinoma of the bladder is one of the most common urological malignancies. An estimated 386,300 new cases and 150,200 deaths from bladder cancer occurred in 2008 worldwide (2) and then numbers are increasing due to the increase in the prevalence of smoking and environmental carcinogens. Smoking cessation programs appear to have reduced, but not completely negated this alarmingly rising incidence (3) The majority of bladder cancer occurs in males and there is a 14-fold variation in incidence internationally. It is four times more common in men compared with women. The mean age is approx 69 years in Men and 71 years in females (4).

Types of cancers of the bladder:

There are various types of carcinoma bladder

1. Transitional cell carcinoma(TCC) of the bladder or the urothelial carcinoma of the bladder
2. Squamous(SCC) cell carcinoma of the bladder
3. Adenocarcinoma of the bladder(5)
4. Lymphoma of the bladder
5. Sarcoma of the bladder(6)

TCC or urothelial carcinoma of the bladder is the most common type of the cancer of the bladder, accounting for approximately 90% of the cancers of the urinary bladder

Classification

TCC of the bladder is broadly classified into two types:

1. Non muscle invasive carcinoma of the bladder
2. Muscle invasive carcinoma

Based upon the **histological** grading, it is divided into:

1. Low grade
2. High grade carcinoma of the bladder.

The WHO recommends(7) the use of the term 'urothelial cancer' (8) as opposed to the more widespread 'transitional cell carcinoma'. The current classification of grade is widely accepted by both urologists and pathologists. Malignant neoplasms are classified as ***low-grade*** and ***high-grade*** (7) depending on the nuclear and cellular architecture. It is the grade,

not stage that is the most important predictor of progression. The low-grade and high-grade tumours can be regarded as distinct diseases with respect to tumour biology, their genetic origin and ultimately management.

Ta – Confined to epithelium

T1 – Invades lamina propria

T2 – Detrusor invasion

T3 – Extravesical spread

T4 – Invasion of adjacent organs

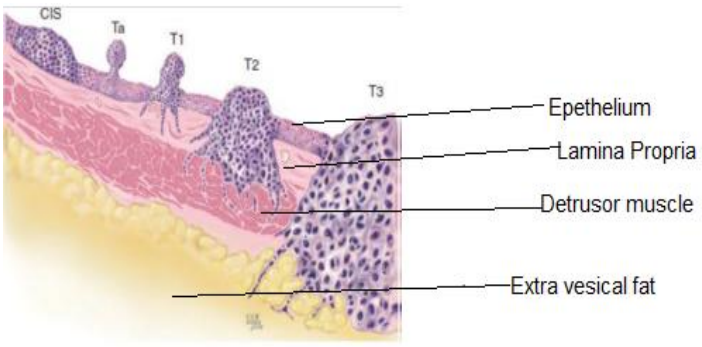


Figure 1: Stages of urothelial carcinoma

The figure 1 diagrammatically depicts the various stage of the urothelial carcinoma of the bladder.

Ta tumours are the ones confined to the epithelial layers and they are usually slow growing and non muscle invasive i.e. basement membrane is not invaded. Their peculiar characteristic is that they may recur many times before they progress to muscle invasive disease or become metastatic (9).

Carcinoma in situ or CIS represents a different entity. Here, there is no papillary tumour but occurs severe dysplasia at the cellular level. If CIS is found next to the area where low grade tumour is present, it behaves more aggressively and there is higher chances of muscle invasion in the future. (10) There have been studies from different centers treating CIS by early radical cystectomy with a definite survival benefit as compared to patients who were

managed with repeated transurethral resections (11). Invasive bladder cancer occurs in more than 75 % of these patients. (12)

T1 tumours involve the lamina propria and the muscle is free. Their treatment varies if they are low grade or high grade, as the low grade tumour usually behave indolent while the high grade T1 tumours are more aggressive and recurrent, requiring aggressive management. These have much higher chances of recurrence as well as progression. (13) Since these have higher propensity of progression and recurrence, these are often initially treated with immunotherapy, mainly BCG(14)

T2 tumours involve the detrusor muscle and are usually treated by radical cystectomy, with or without adjuvant therapy (7). Long term cancer free results can be available if radical cystectomy is done at a stage when it is amenable to cure.(15)

T3 tumours extend beyond the bladder i.e. extravesical disease. (16). Various targeted therapies are available to downstage the disease before radical cystectomy (17).

T4 tumours invade the adjacent organs like prostate, seminal vesicles, pelvic bones etc. The survival of these patients is uniformly poor. Even after the availability of various chemotherapy regimens, the survival has not improved much. Efforts have been made to look upon various biochemical pathways and identify genetic lesions so that in future the disease is arrested in the earlier stages or better modalities are offered (18).

Bladder cancer or the urothelial cancer is called a multifocal disease due to the theory of field cancerization i.e. the tumour tends to recur at the sites away from the original site where it was present earlier. But the genetic studies give a contrary view of its being of monoclonal origin. Simon et al studied 32 tumours from cystectomy specimens and found 17p losses in all tumours along with a very high numbers of chromosomal changes. They also found an identical TP53 mutation in all the tumours as well as the adjoining normal looking mucosa. They hypothesized that the tumour cells carry a specific chromosomal aberrations at the intraepithelial level thus suggesting a monoclonal origin of the multifocal bladder tumour (19).

Most of the carcinogens in the body are detoxified and excreted in the urine and hence the urothelium is exposed to various carcinogens. This explains the multifocal occurrence of the various urothelial tumors in the upper urinary tract and the bladder.

Etiology and the risk factors for TCC bladder (20)**:** Environmental risk factors and genetic factors

1. Smoking, both active and passive smoking.
2. Occupational carcinogens
3. Genetic effects

Smoking: Smoking is an established risk factor for transitional cell carcinoma of the bladder. For active smokers, the risk is approx 4 times as compared to non-smokers. If a person stops smoking, though the risk decreases gradually, it doesn't touch the baseline even after 20 years.(21)

Even passive smoking is associated with increased risk of TCC bladder and other cancers.(22) Urothelial tumours in smokers are usually of higher grade and they have worse prognosis.(23) Apart from these, smoking also decreases the general health of the patient and it is one of the commonest factor which delays the curative surgical management of the cancer patients. The trend towards smoking is increasing in the urban and the younger population.

Occupational carcinogens: Metal workers, rubber industry workers, leather workers, miners, cement workers, painters, plastic and other industrial workers. Bladder cancer also has been associated with exposure polycyclic aromatic hydrocarbons (PAHs), paint components, and diesel exhausts. (24)

Miscellaneous risk factors known to cause TCC of the bladder are chronic cystitis, HPV infection(25), bladder augmentation, neurogenic bladder (26), upper urinary tract cancers, radiation to the pelvis, chronic analgesics and cyclophosphamide chemotherapy.

Genetic effects:

TCC has long been known to cluster in families suggesting a role for genetic factors. Studies by Aben et al and Goldar et al have shown increased risk of TCC bladder in the relatives of the patients with TCC (27,28). In literature there are reports of two successive generations being affected suggesting an autosomal dominant pattern (29) How ever though Mendelian pattern of inheritance has been spoken about, contrary to the belief second and third degree relatives had higher observed to expected ratio of TCC than first degree relatives yet again suggesting other modes of inheritance. As per large scale studies inheritance clearly appears to be multifactorial. The risk was more in relatives who were smokers than in non

smokers. These patients also have bladder cancer at a much younger age than with no family history of TCC bladder; however some studies have not found the same.

Complex interactions of susceptibility genes, environmental risk factors leading to activation of Oncogenes and suppression of tumour suppressor genes leading to carcinogenesis in the urothelium has been suggested.

Susceptibility for bladder cancer has been studied using Genome Wide Association Studies [GWAS], which suggested an association with genes like TACC3 (30) as well showing some association with few SNPs upstream of MYC gene and TP53 (31). The natural history of the TCC bladder can get altered due to the complex interactions of these factors.

It has been also suggested that genetic factors increase the risk of TCC bladder by altering the ability to detoxify carcinogen precursors however GWAS did not show any hits in the genomic regions having genes for the xenobiotic enzymes.

Pathogenesis:

As described above, the strong association of TCC with tobacco smoking is due to various carcinogens in the tobacco smoke. Cigarette might look harmless in a white classic paper, but when it burnt releases many harmful chemicals, which can cause cancers of the various parts of the body

Cancer causing agents in cigarette smoke are(32)**: Tar, arsenic, cadmium, formaldehyde, benzene, polycyclic aromatic compounds, nitrosamines, Acrolein, cadmium, carbon monoxide, ammonia and many others.**

Most of the above mentioned carcinogens, apart from heavy metals are detoxified by series of xenobiotic enzymes. The xenobiotic metabolizing enzymes are divided historically into phase I enzymes, which are exclusively cytochrome P-450 dependent and phase II enzymes, such as Glutathione transferase (GST) and sulpho-transferase. These carcinogens are mainly detoxified by phase II metabolic enzymes like Glutathione S transferase (33,34). These are involved in N-oxidation to N-hydroxylation metabolites.

These enzymes are polymorphic i.e. there are variants with slightly different molecular structures and hence differential biologic activities. There are many Case-control studies suggesting that cigarette smokers with different polymorphisms have a differential risk for bladder cancer.(35)

Various studies have looked at GSTP and GSTM polymorphisms and their association with TCC bladder. The results of studies have been conflicting, though majority of studies have found significant associations with particular polymorphisms (36). It is generally accepted that overall GST activity is also up regulated in transitional cell carcinoma (TCC) of the urinary bladder and GSTP1 accounts for the majority of this increase(37).

The polymorphisms mentioned above, lead to over excretion of the aromatic amines and polycyclic hydrocarbons, causing oxidative damage to the DNA of the bladder epithelium there by leading to urothelial carcinoma of the bladder (38).The DNA damage leads to activation of oncogenes and suppression of tumor suppressor genes resulting in unregulated clonal proliferation. The same has been examined in few studies. Role of TP53 has also been studied; it is a cell cycle regulator, a meta-analysis of 168 studies done by Malat et al showed a significant association of p53 over expression in TCC bladder (39).

Approximately 50% of the bladder tumour has somatic mutations in the TP53 gene (40–42). Retinoblastoma gene is also involved in controlling the cell division process. Alteration in this gene is also associated with TCC bladder.(43)

Figure 2: Hypothetical diagram of a gene

The gene (Fig 2) was defined classically as a segment of DNA encoding a protein (44). Various genes are present in the double helix DNA. In a particular gene, two regions are present called Introns and the Exons. When the DNA is transcribed into RNA, the introns are the regions which are removed in the splicing process while the remaining Exons encode the protein. The DNA replication is a highly accurate process but rarely errors in DNA replication can occur, causing spontaneous alteration in the base sequence of the particular gene. These errors are called mutations and they do affect the phenotype occasionally.

Genotype: The combination of two alleles at one genomic location and/or base pair in an individual

Polymorphism: in simple words, it is the occurrence of more than one form or morph.

Genetic polymorphism is defined as a variant of alternative DNA sequence or chromosome copy number that can be found in a population.

Genetic polymorphism: definition as given by Phillip Hedrick, is the occurrence in the same population of two or more alleles at one locus, each with appreciable frequency", where the minimum frequency is typically taken as 1%. (45) It is hypothesized that the polymorphism might alter a particular enzyme's function and makes it weaker or more potent.

GLUTATHIONE S TRANSFERASE

Structure and types of GST

The complete human genomic glutathione-S-transferase-pi gene (GST-pi) was isolated from a lambda Charon 4A bacteriophage library which was screened by hybridization to a human GST-pi cDNA. The GST-pi gene has 7 exons and 6 introns contained within approximately 2.8 kilo-bases.(46)

The mammalian GST enzyme superfamily consists of cytosolic dimeric isoenzymes of size 45-55KDa. Till date, human cytosolic GST enzymes are subdivided into 8 subtypes: alpha, mu, kappa, omega, pi, sigma, theta and zeta. This classification is based on immunological cross-reactivity and on the basis of sequence similarity. The structure and the mechanism of

glutathione-S-transferase are studied by site directed mutagenesis and X-ray crystallography (47). These subtypes are encoded by the genes GSTA, GSTM, GSTK, GSTO, GSTP, GSTS, GSTT and GSTZ, respectively. But among these, the functional polymorphism is only indentified in the GSTM1, GSTP1 and GSTT1 gene (46,48).

GSTP1 is the most commonly studied enzyme amongst all these. It is located on chromosome 11q13. It is well recognised and is proved in various studies that GSTP1 plays an important role in protecting cells from various carcinogenic as well as from cytotoxic agents. It is well expressed in different cell types, as described above. GSTP1 DNA hypermethylation at the CpG island in the promoter-5' region can result in the altered or decreased GSTP1 activity (49).

Functions of Glutathione S Transferase

GST enzymes are the major phase II detoxifation enzymes found mainly in the cytosol in mammals (50). It catalyses the conjugation of the reduced glutathione (GSH) with the compounds that contain an electrophilic centre through the formation of the thioester bond between the sulphur atom of GSH and the substrate(51). Other than conjugation reactions, GST enzymes also have non-catalytic functions like intracellular transport of hydrophobic ligands, segregation of carcinogens and modulation of signal transduction pathways.(52)

Below mentioned (Figure 3) is the hypothetical natent structure of the GSTP1 protein and if at 313 position of DNA, there is a change of nucleotide Adenosine to Guanine, the isoleucine at 105th position of the protein changes to valine and it has been suggested that this leads to structural alteration in the protein causing diminished enzymatic activity. The introduction

of valine is hypothesized to alter the protein to such a level that it responds less to the toxins in the body and thus predisposes the individual to develop cancer.

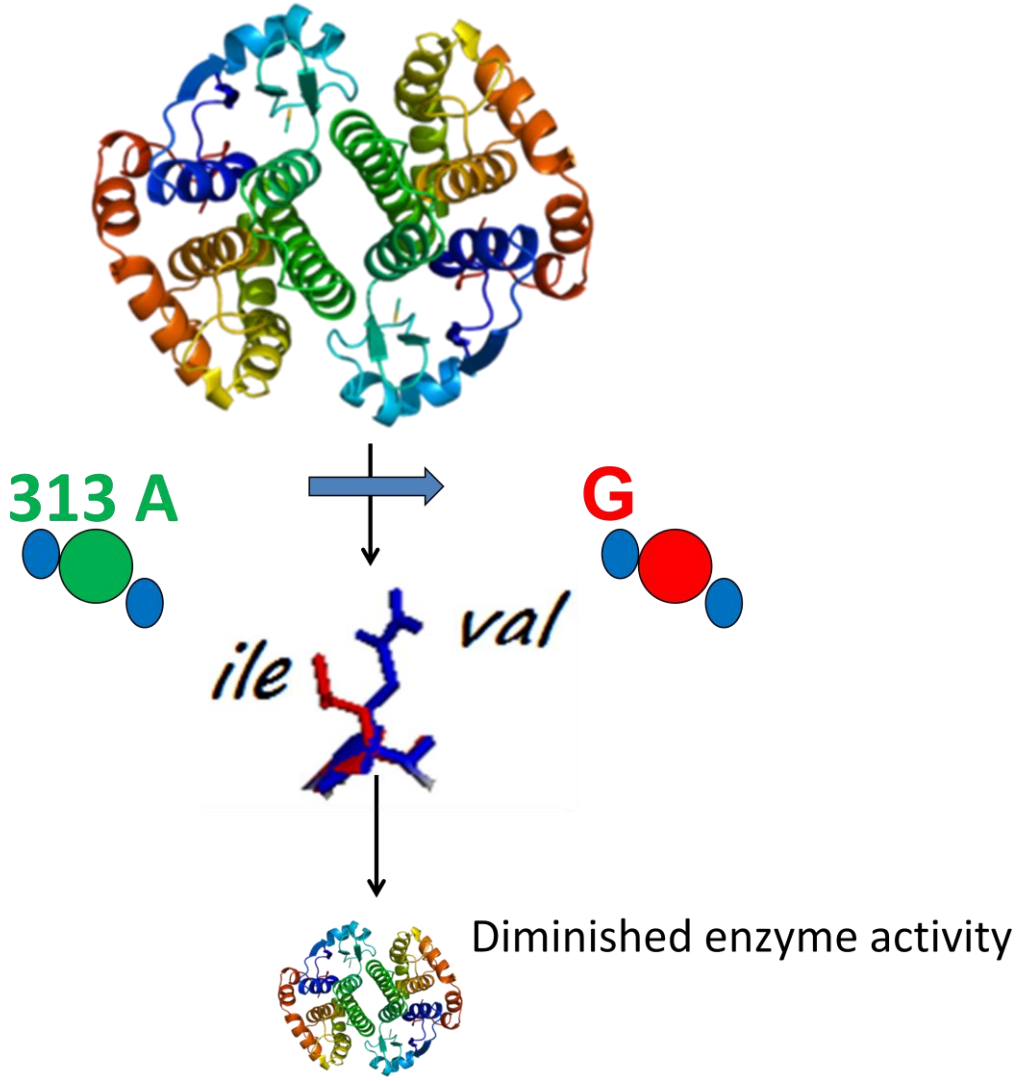


Figure 3: Figure depicting the hypothetical natent structure of GSTP1 protein and the changes occurring which causes the protein to have diminished activity.

This substitution leads to three GSTP1 genotypes: Homozygous wild type isoleucine/ isoleucine allele (ile/ile), heterozygous isoleucine /valine allele (ile/val) and homozygous polymorphic valine/valine allele (val/val)

Various biochemical studies indicate that GSTP1 polymorphic valine allele has a lower thermal stability than GSTP1 homozygous isoleucine wild allele. The valine allele has a lower conjugating activity and the heterozygous isoleucine/ valine allele has an intermediate thermal stability.

Table 1: Normal tissue distribution of GSTP1

Super family	Class	Chromosome	Protein	Organs
Soluble	Pi	11q13	GSTP1	Brain>heart=lung=testis>kidney=pancreas

The table 1 depicts the normal distribution of GSTP1 protein in the body. Pi class of glutathione-S-transferase is located on chromosome 11q13 (53). It is expressed in normal tissues at variable concentration in the body with the maximum amount in the brain and in the heart, lungs, testis, kidneys and the pancreas In the decreasing order respectively.

Although there are various polymorphism described in GST genes, the physicochemical approaches to detect polymorphism were limited to analysis of three Classes Alpha, Mu and Pi isoenzymes.

Table 2: Polymorphic GSTP1

Class	Gene	Allele	Alteration in gene or in nucleotide	Protein or amino acid affected
Pi	GSTP1	GSTP1 A	A313,C341,C555	Ile *105*, Ala *114*, Ser *185*
		GSTP1 B	G313,C341,T555	Val *105*, Ala *114*, Ser *185*
		GSTP1 C	G313.T341,T555	Val *105*, Val *114*, Ser *185*
		GSTP1 D	A313,T341	Ile *105*, Val *114*

Four different GSTP1 allele have been identified according to the SNPs at different positions in the open reading frame of GSTP1 gene leading to the substitution of amino-acids at 105 and 114 as illustrated in the table above (Table 2). These polymorphisms make significant inter-individual difference in the drug or toxin metabolism. But polymorphism in individual genes does not obviously confer a markedly increase in risk of the cancer. In various studies, typically odds ratio associated with any single variant GST allele and the development of particular neoplastic disease are found to be less than 3.0(54)

The GST polymorphism not only appears to influence susceptibility to disease and but also influences the responsiveness to various carcinogens. Apart from urothelial cancers, GST polymorphism has been implicated in various cancers like hepatocellular carcinoma, breast cancer, prostate cancer, renal cancers, testicular cancers etc.(55)

Various studies imply that even with occupational exposure, GSTP1 polymorphism is associated with increased risk of DNA damage. (56)

Methods involved for studying polymorphism in genes

1. **DNA extraction**
2. **Polymerase chain reactions**
3. **Restriction fragment length polymorphism**
4. **Gel casting**
5. **Electrophoresis**

DNA extraction or Isolation:

DNA extraction is the process of purifying DNA from sample. It involves both physical methods and chemical methods. It was first described by Friedrich Miescher in 1869. It is a routine method used in molecular biology or forensic analyses.

Basic procedure of DNA extraction:

The following steps are used for DNA extraction:

The cells consist of biomolecules namely nucleic acids, proteins, carbohydrates and lipids. Other molecules have to be removed to isolate the nucleic acid.

First step is to lyse the RBCs using the RBC Lysis buffer. The remaining lysate consists of WBCs which are disrupted using cell lysis solution – a detergent (SDS). The cellular and histone proteins which are bound to DNA are denatured and precipitated using protein precipitation solution (Proteinase K an enzyme). This precipitated solution is treated with Phenol-chloroform to lyse the carbohydrates and lipids which then along with the lysed protein separate into aqueous phase and organic phase, the DNA suspended in the aqueous phase. Suspended nucleic acid is precipitated using 100% ethanol. The remaining salts are

removed by washing the nucleic acid with 70% ethanol twice or thrice. The DNA is allowed to dry and is resuspended in Tris-EDTA buffer (TE).

TE buffer comprises of Tris buffer and EDTA that chelates magnesium ions. TE buffer prevents DNA degradation.

Polymerase chain reaction (PCR): PCR was developed by Kary Mullis in 1983. It is the technique which involves enzymatic exponential amplification of DNA generating thousands to millions of copies of the target DNA sequence.

PCR Method depends upon thermal cycling (Peltier effect) i.e. repeated cycles of heating and cooling of DNA to attain the required temperature for the successful DNA amplification.

The two key components involved are the short single stranded oligonucleotides (DNA fragments 18nt to 20nt in length) or the primers which are complementary to the target DNA sequence and a proof reading Taq DNA polymerase enzyme which catalyses the enzymatic amplification. As the reaction continues, DNA gets amplified in an exponential pattern and the product obtained at the end of each cycle acts as the template for the next cycle.

Steps of PCR

1. DNA template containing the specific DNA region to be amplified.
2. Forward and reverse primers complementary to the sense and anti-sense strand of the DNA target.
3. Taq DNA polymerase with a temperature optimum at 72 °C.

4. dNTPs (deoxynucleotide triphosphates - building-blocks) which the DNA polymerase utilizes to synthesis a new DNA strand.
5. A Buffer solution to maintain proper chemical environment for optimal enzymatic activity and to stabilize DNA polymerase
6. Divalent cations (magnesium ions) acts as a cofactor to the DNA polymerase
7. Monovalent cation potassium ions are used for DNA stabilization.

Small reaction tubes of 0.2-0.5 ml are used to carry out PCR. These tubes are kept in a thermal cycler, which alternatively heats and cools the reaction tubes to achieve the desired temperature.

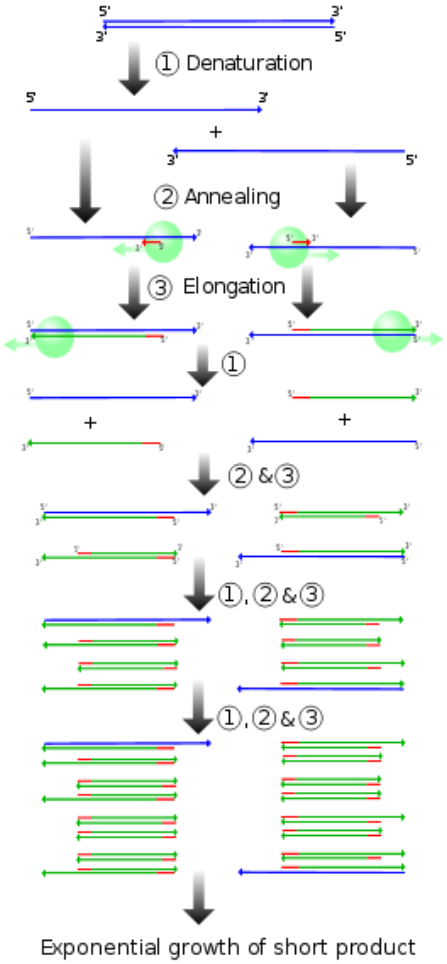
Procedure of PCR: PCR consists of approximately of 20-40 repeated temperature cycles in the thermal cycler

1. Denaturation step: In this step the genomic DNA double helix are physically separated into two strands. This involves heating the sample at 94–98 °C for 5min. Longer duration is given to achieve complete denaturation of the genomic DNA. At high temperature the H bonds between the nucleotides break and the DNA attains single stranded conformation. However subsequent denaturation steps are done for lesser duration (1 min) since this time the target region has to be separated into single stranded.
2. Annealing step: During this step the primers get annealed to their complementary sequences in the target region. The optimal annealing temperature is 50–65 °C for 20–40 seconds. The primers form H bonds with their complementary nucleotide. The

Taq polymerase binds to the primer-template complex which is now ready for extension of the target region.

3. Extension/elongation step: The extension temperature depends on the type of DNA polymerase. Taq DNA polymerase required 72°C for extension. The time required for extension depends on the length of target region. The optimal time is 1 min for extension of 1000bp target length. DNA polymerase utilizes the dNTP which are added to the 3 prime end of the primer and the fragment is extended.
4. Final elongation: This is performed at a temperature of 72 °C for 5–15 minutes after the last cycle of PCR. This ensures that all the end fragments are completely extended.
5. Final hold: This step is applied for short-term storage of the reaction for an indefinite time

Figure 4: Schematic drawing of the PCR cycle.



Restriction fragment length polymorphism

In RFLP analysis, the DNA fragment is restricted (digested) by restriction enzymes (endonucleases) and the fragments thus obtained are separated according to their respective lengths (base pairs) by gel electrophoresis

Restriction enzyme:

The PCR product is digested with the restriction enzyme called Alw26I. It is a fast digest enzyme for rapid DNA digestion. It recognizes and cleaves the DNA wherever a specific short sequence (usually palindromic) occurs. The *Alw26I* restriction enzyme recognizes 5'GTCTC3' sites and cuts best at 37°C overnight. Fast digestion is done at 56°C for 1 hour.

RECOGNITION SITE:

5' G T C T C N↓ N N N N 3'

3' C A G A G N N N N N↑ 5'

Gel electrophoresis: Based upon the size and the charge, DNA or RNA is separated for analysis using an electric field by a method called electrophoresis. They can be made to migrate through the pores of an Agarose gel or polyacrylamide gel. After connecting to the power source (120V and 400mA) the gel is placed in an electrophoresis chamber. Now the electric field is applied to move the negatively charged DNA molecules through the agarose matrix from cathode to anode.

DNA is loaded in wells of the gel and molecules migrate through the matrix at different rates. The smaller fragments migrate easily through the pores of the gel compared to the longer or larger fragments. Hence different sized DNA fragments are seen as distinct bands which can be easily read. Often many numbers of samples are loaded simultaneously in the adjacent wells. Hence fragments of similar weight move together, parallel in their individual lanes and will be at a uniform distance from the wells.

Types of the Gel

1. Agarose
2. Polyacrylamide
3. Starch

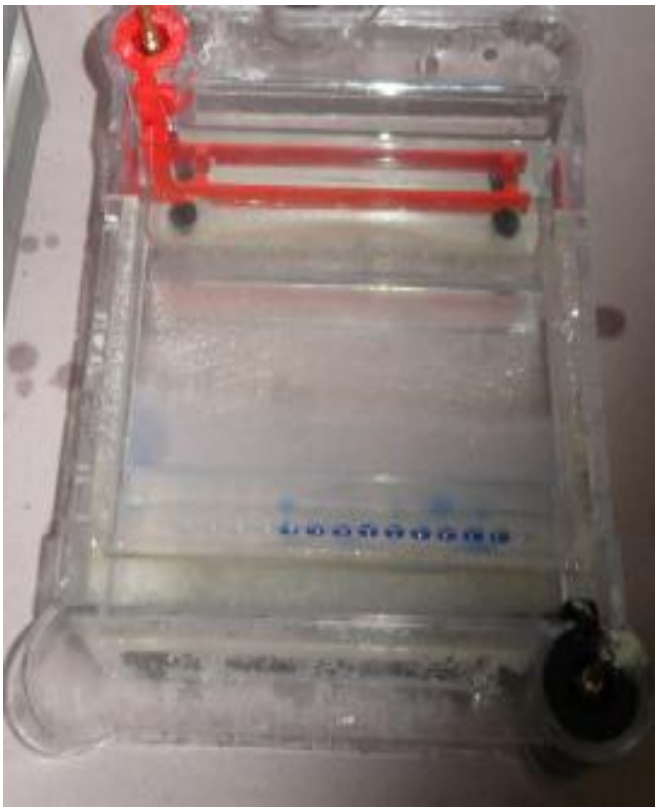
Agarose gel: Although they do not have uniform pores, they are optimal for electrophoresis of DNA. The percentage of the agarose in the gel determines the distance travelled by different molecules of the DNA. Larger fragments of DNA are separated in lower percentage of agarose to facilitate the movement of DNA through small pores and smaller sized fragments are separated in higher percentage of agarose. The agarose gel is easy to made, stable and can be handled easily as compared to other gels. The remaining gel can be stored easily in the refrigerator, to be used again. In case the gel is of high percentage of agarose, the use of pulsed field electrophoresis or field inversion electrophoresis can be used to hasten up the speed of electrophoresis. These gel are often brittle and do not set easily. Low percentage gel is very weak and breaks when lifted. 1% agarose gel is often used commonly in genetic studies.

Buffers: Ions in the buffer carry current and facilitate the DNA movement from cathode to anode. They also maintain constant pH. Most commonly used buffers are Tris Acetate EDTA (TAE) and Tris Borate EDTA (TBE)

Visualization: The molecules in the gel are fluorescently stained to make them visible under UV after the electrophoresis. Ethidium bromide is used for fluorescently staining DNA. Ethidium bromide is carcinogenic; care is required to handle it. Ethidium bromide intercalates into DNA and is perpendicular to the axis. It emits radiation energy at 590nm

under ultraviolet light. This makes the visualization very clear. Gel documentation Unit takes the photographs which are then studied for final results. Shown below (figure 5) is the gel electrophoresis picture.

Figure 5: Gel electrophoresis picture



Various studies have shown that glutathione S transferase is not only involved in urothelial cancers, but also in the renal cancer and the cancers of the prostate (55). In the presence of risk factors, although the prevalence of Urothelial carcinoma of bladder is higher, not all exposed individuals are affected with cancer. Thus it is hypothesized that polymorphism in the group of genes, resulting in decrease in the level of GST enzyme, may lead to increase risk of urothelial cancers.

Since the discovery of the potential association of GSTT1 and bladder cancer in 1996, various studies have investigated the association between TCC bladder and GSTs genes. Many studies have seen the relationship of TCC bladder with the polymorphism of GSTP1 gene along with GSTM1 and GSTT1 polymorphism reported that in Turkish population (57), GSTP1313 A/G or G/G genotype increases the susceptibility of urothelial cancer by 1.75 times. Srivastava et al (58) in the north Indian population 2005 reported a strong association of homozygous polymorphic valine allele and urothelial cancer of the bladder. They reported more than 7 times increased association with GSTP1 polymorphism and urothelial cancer of the bladder. He also proved that if the three genotypes of GST are combined, the gene-gene interaction further increases the risk of urothelial cancer of the bladder.

Safarinejad MR et al in 2011 suggested that polymorphism of GSTP1 along with GSTM1, and GSTT1 may be associated with increased bladder cancer risk in the Iranian population(34) Ercegovac MP et al in 2008 concluded that along with increased GSTP1 over expression in transitional cell carcinoma patients, altered apoptotic pathway was also seen.(59)

But Goerlitz D et al in 2011 did not find any association of TCC bladder with GSTP1 polymorphism. They suggested no association with Genotypes and smoking, environmental toxins or infection.(60)

Similar observations were seen by Katoh T et al(61) in Japanese population, they found no association of between valine polymorphism in control or cancer population. They also found no association of polymorphism between smokers and never smokers in any of the cancer group, oral, lung, gastric or urothelial cancers

Steinhoff C et al in German population(62) also suggested no correlation between GSTP1 polymorphism and Urothelial carcinoma of the bladder. Pandith AA et al(63) also found no statistical significance difference in the genotype frequencies between the cancer and the control population in Kashmir Indian population. They also found a significant risk of more than 2.5 times for the polymorphic allele (AG+GG) with smokers in cases as compared to controls ($p<0.05$)

Hence the results in these epidemiologic studies in literature were very inconsistent and conflicting with some studies showing increased risks and the other showing no association with bladder cancer and GSTP1 polymorphism

The purpose of our study was to determine the frequencies of polymorphisms of GSTP1 genes and their association with TCC bladder among the patients presenting to a tertiary care urology centre.

Material and methods

This was a prospective study done at the Department of Urology and Medical Genetics of a tertiary care hospital, for a period of one year from August 2012 to July 2013. It was approved by the institutional review board, (Institutional research board no: 8063/6.11.2012).

Subjects- Cases and Controls

This study consisted of total 98 patients, 52 patients with histo-pathologically confirmed diagnosis of Primary TCC bladder (mean age ± SD, 56.2 ± 10.6) and 46 cancer free controls who presented with non-malignant urological problems (Mean age: 52.7± 13.2)

Criteria for the patient selection were based on a questionnaire covering medical, pathological, and histo-pathological records in Christian Medical College and Hospital (CMC) Vellore. All patients were carefully paired with controls to avoid any bias or confounding factors. They were selected in regards to Age, smoking or tobacco chewing habits, occupational exposure, diet factors, family history of cancers and smoking awareness. Since CMC caters to a wide variety of patients from different ethnic origin, both the cases and controls were carefully matched for their ethnic origin. No patient had a prior history of chemotherapy or radiotherapy before recruitment because it is a well known that chemotherapy and radiotherapy can cause damage to the DNA, resulting in false and misleading results. Majority of the controls were the patients undergoing treatment for urethral stricture disease or the renal/ ureteric stones and they underwent cystoscopy to rule out obvious urothelial carcinoma of the bladder.

Informed consent was taken from all the subjects before they participated into this study. This was conducted according to Helsinki declaration (2004).

Inclusion /Exclusion criteria: Case group were the patients with the diagnosis of Primary TCC bladder. All patients who had metastasis to bladder or with previous history of radiotherapy or chemotherapy were excluded.

In the control groups, we took patients with non-malignant urological diseases and without history of any other malignancy in the past and in cystoscopy, no evidence of TCC was seen.

Evaluation: All subjects were asked relevant history and they had a complete physical examination and answered a structured proforma that included demographic background, smoking awareness and smoking habits, occupational history, Chronic disabling illness, personal and medical history.

We considered the subject as a non-smoker, if he has not smoked for the last 10 years. Only the subjects with the biopsy proven TCC bladder were taken, either after the transurethral resection of the bladder tumor or after the radical cystectomy. The tumors were classified as Non-muscle invasive Bladder (pTa–pT1) or muscle invasive (pT2–pT4) according to the TNM staging system of the American Joint Committee on Cancer.

Table 3: TNM staging of the Transitional cell carcinoma of the bladder

Ta	Papillary, epithelium confined
Tis	Flat carcinoma in situ
T1	Lamina propria invasion
T2a	Superficial muscularis propria invasion
T2b	Deep muscularis propria invasion
T3a	Microscopic extension into perivesical fat
T3b	Macroscopic extension into perivesical fat
T4a	Cancer invading pelvic viscera (e.g., prostatic stroma, vaginal wall, rectum, uterus)
T4b	Extension to pelvic sidewalls, abdominal walls, or bony pelvis
N0	No histologic pelvic node metastases
N1	Single positive node ≤2 cm in diameter, below common iliacs
N2	Single positive node 2-5 cm in greatest diameter or multiple positive nodes
N3	Positive nodes >5 cm in diameter
Nx	Nodal status unknown
M0	No distant metastases
M1	Distant metastases documented
Mx	Distant metastases status uncertain

The histological grades were subdivided into low grade and high grade according to the WHO classification (WHO) and the International Society of Urological Pathology (ISUP)

Genotyping and Method of estimation: The GSTP1 genotype was determined by PCR-restriction fragment length polymorphism (RFLP) technique.

For the extraction of genomic DNA from the blood sample, the RBC are lysed and DNA extracted from the WBC. They are centrifuged and pellets are made. As shown in the figure 6

Figure 6: Centrifugation of the serum

Figure 7: Amplification is done in the PCR machine:

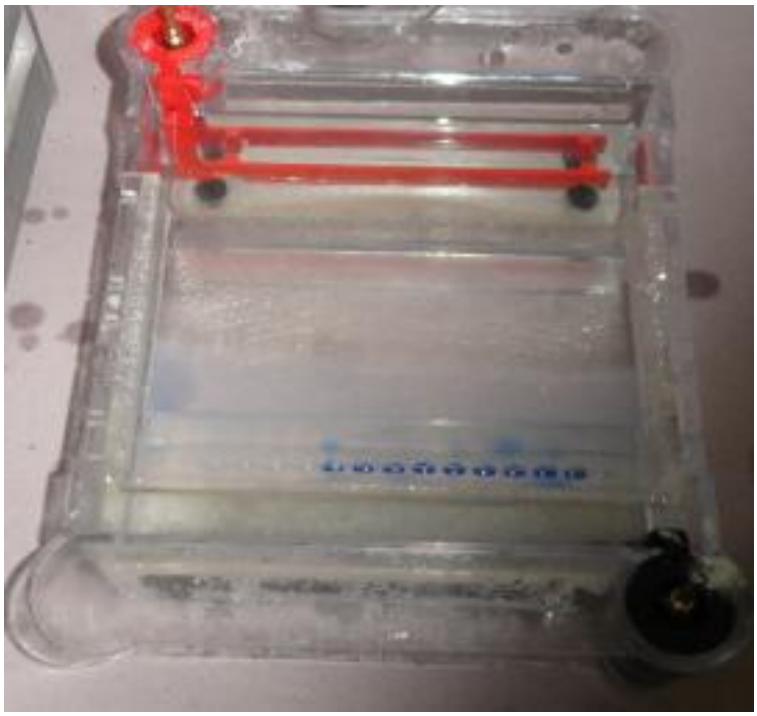
Using the primer pair, a 177 bp fragment of the GSTP1 gene containing Ile to Val substitution in exon 5 (codon 105) will be amplified (Figure 7)

Forward: 5'-ACC CCA GGG CTC TAT GGG AA-3' and

Reverse: 5'-TGA GGG CAC AAG AAG CCC CT-3'.

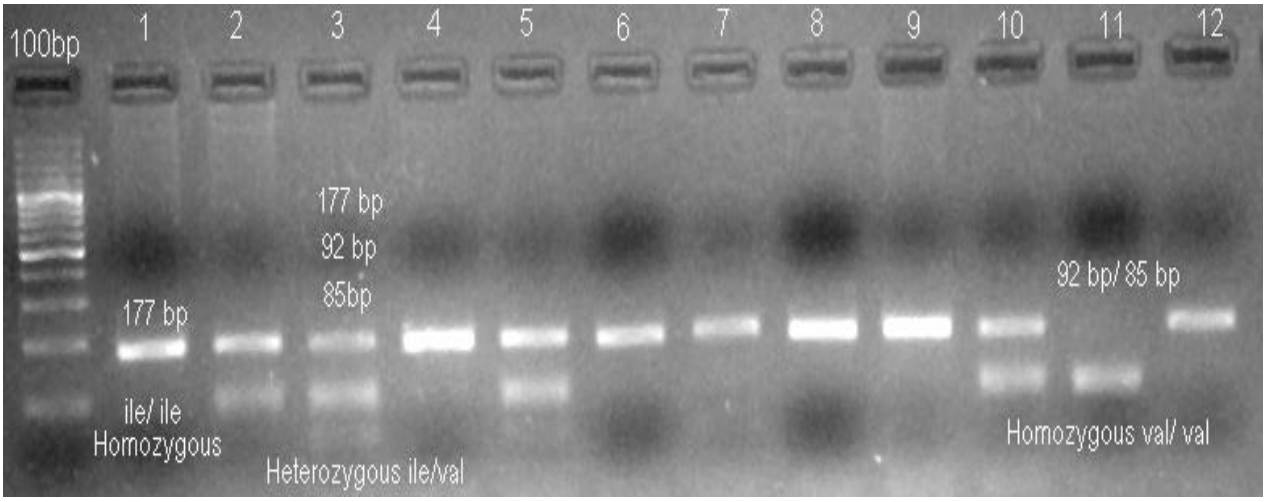
The amplification cycles included an initial denaturing at 94°C for 30 seconds, followed by an annealing step at 55°C for 30 seconds, and a final extension step at 72°C for 30 seconds, a slight modification from the method described by Safarinejad MR et el. The PCR products then will be digested with *Alw*26I restriction enzyme.

Figure 8: Gel electrophoresis picture



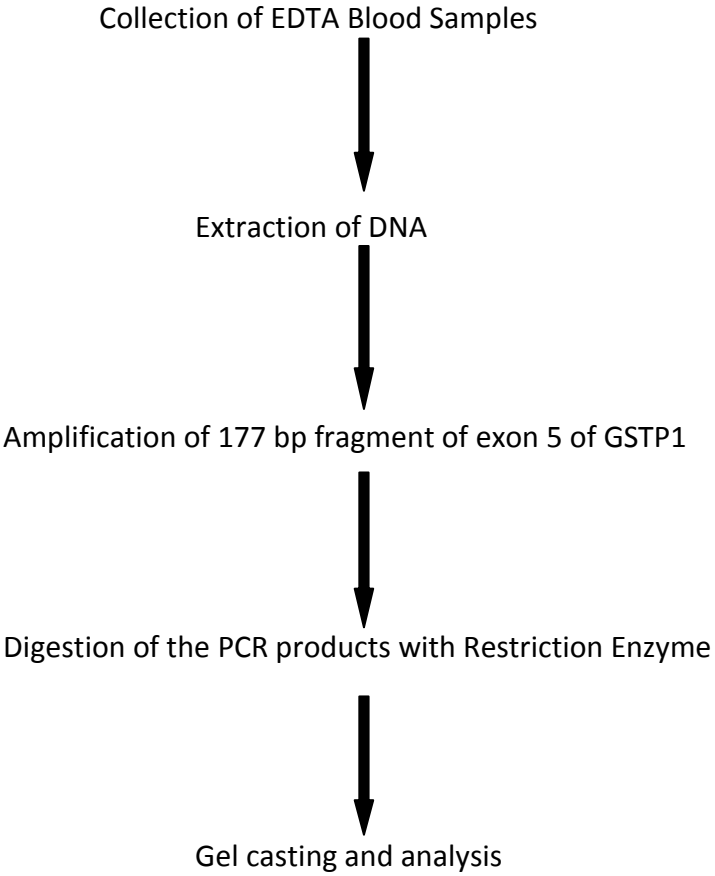
Dye is added to the Restriction products and they are analyzed by 2% agarose gel.

The PCR product (Figure 9):



Homozygous Ile/Ile individuals will have a single fragment of 177-bp (base-pairs) and it appears as a dark band, and homozygous Val/Val individuals will have both 92- and 85-bp fragments- appearing as a faded band. The presence of all 3 fragments will correspond to heterozygous Ile/Val individuals.

FLOW CHART



Quality control: To maintain a good quality control, 10 % of the duplicates for both the cases and the controls were genotyped with 100% concordance to the genotype by PCR-RFLP

Even the laboratory personnel were not able to differentiate between the cases and control samples.

Statistical methods: The power of the study – 80% was determined considering of following variables:

1. Case-control study design
2. Significance level 5 % (2 sided)
3. Expected frequency of risk allele in the control population = 0.28
4. The genetic effect for odds ratio (OR) 2.5 or greater.

Assuming log-additive model of inheritance and frequency of risk allele to be 0.28 [-ref], a total sample size of 98 (52 cases/ 46 controls) would be required to detect an odds ratio of 2.5 or more with a desired power of 80% and a significance level of 5%

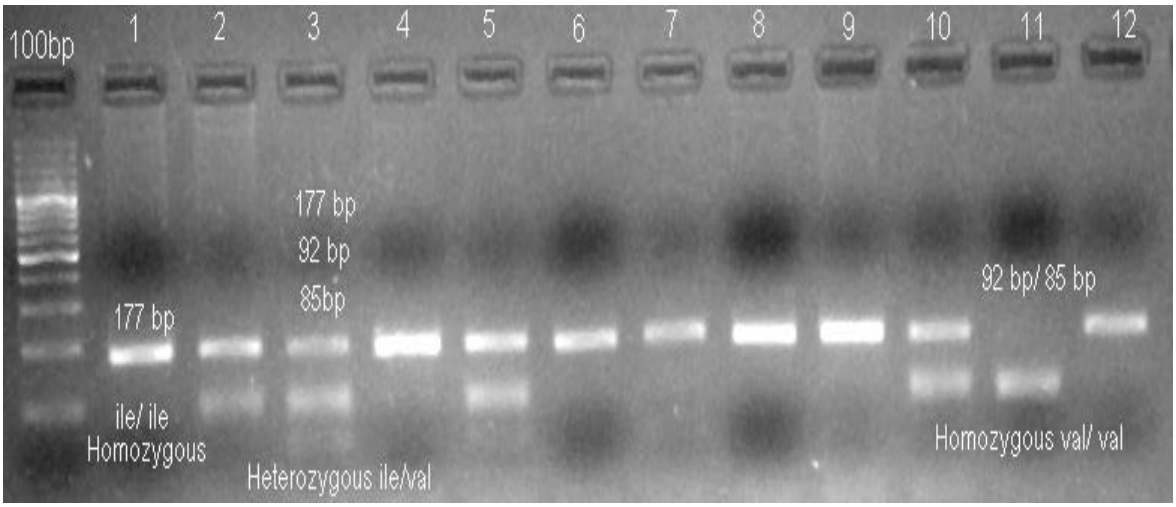
Results are expressed as mean ± standard deviation (SD).

Hardy-Weinberg equilibrium was examined using the goodness-of-fit X^2 test. It is done to compare the observed allele frequencies in the study with the expected frequencies determined from control subjects.

To assess the homogeneity between cases and controls regarding their geographic origin, smoking awareness, cigarette smoking, disease pathological status and genotypes, the X^2 or Fisher's (F) exact test was used.

To determine the association of polymorphisms with susceptibility to develop TCC and between the various clinicopathological characteristics of bladder cancer, we used unconditional logistic regression to estimate adjusted odds ratios and 95% confidence intervals (CI). Statistical Package for the Social Sciences (SPSS 18) was used for statistical analysis.

Figure 9: Representative PCR-RFLP products



Restriction-digestion of The PCR products of GSTP1 gene by the *Alw26I* restriction endonuclease.

Results

Characteristics of the study population

Table 4 shows the demographic and clinical characteristics of both the cases and the controls

Both the groups are matched on the basis of Age, gender and geographic distribution. There was no confounding factor seen.

Table 4

Demographic characteristics of the cases and controls				
Characteristics		Cases (n-52)	Controls(n-46)	P values
Age(yrs)		56.2± 10.6	52.7± 13.2	0.151
Gender	Male	45	40	
	Female	7	6	0.950
Origin	North	11	7	
	North-East	30	25	0.511
	South	11	14	
Smoker	Yes	32	12	
	No	20	34	
Awareness- smoking causes bladder cancer	Yes	30	44	
	No	20	2	
Occupational exposure	Yes	19	8	

As shown in the table 4, the mean age of the cases were 56.2± 10.6 years and the mean age of the controls were 52.7± 13.2 years (p value- 0.151)

Majority of the patients, in both the cases as well as the control groups were males. (p value- 0.950) Since our hospital caters to population all over India, we had a heterogeneous population with majority of our cases and controls coming from the eastern part of India. While for the northern and the southern parts, the cases were almost equally distributed. (p value-0.511). 72.7 % of all the smokers had TCC bladder. It was interesting to note that 66.6 % of the cases were aware of the fact that smoking causes TCC bladder but they still continue to smoke

36.5 % of our cases and 17.3 % of our controls were exposed to various forms of toxins due to their occupation. But this difference was not statistically significant.

Table 5

The clinicopathological characteristics of Transitional cell carcinoma (n=52) patients		
Variable		Mean ± SD or n(%)
Age(yrs)		56.2 ± 10.6
Tumor stage	Non-muscle invasive (pTa- pT1)	40(76.9)
	Muscle invasive(pT2-T4)	12(23.7)
Pathological grading	Low grade	11(21.1)
	High grade	41(78.8)
Tumor number	Single	32(61.5)
	Multiple	20(38.5)

As shown in the table 5, the mean age of the cancer patients were 56.2 ± 10.6 years. Majority (76.9%) of the patients were having non-muscle invasive disease. The patients who had muscle invasive disease ultimately underwent radical cystectomy and were cured of the disease.

In 78.8 % of tumour was of the higher grade. 72.7 % of all the smokers had TCC bladder and amongst them 75 % had the disease of higher grade which is much more aggressive. 61.5 % of the patients had single tumour in the bladder

Genotyping

Table 6

The Genotype distribution of the GSTP1 polymorphism between the cases and controls			
Genotype GSTP1	**Cases(52)**	**Controls(46)**	P value
ile/ile (AA)	33(63.4%)	31(67.4%)	.202
Ile/val (AG)	18(34.6%)	11(23.9%)	
val/val (GG)	1(1.9%)	4(8.7%)	

The GSTP1 genotype was in Hardy-Weinberg equilibrium for the controls ($X^2 = 0.68$)

The frequency of both the homozygous wild type and homozygous Polymorphic allele were more in the Control group. We noticed that the predominant allele in both the cases and controls were the wild type i.e. isoleucine- isoleucine i.e. 63.4% and 67.4% respectively

It was interesting to note that the heterozygous polymorphic allele (AG) was seen more frequently in the cancer patient group as compared to the control group i.e. (34.6% vs 23.9%).

However, there was no statistically significant difference seen in the allele distribution among cases and control (p value- .202), suggesting the lack of association of the homozygous or heterozygous valine allele with TCC bladder

Subset analysis of the genotype frequencies- For geographic distribution i.e. North Indians, East Indians and South Indians

Table 7

The distribution of GSTP1 genotype according to the geographic origin

7a

		Ile/ile(AA)(14)	**Ile/val(AG)(4)**	**val/val(GG)(1)**	P value
North-Indians	**Cases**	8	2	0	1.00
	Controls	6	2	1	

7b

		Ile/ile(AA)(34)	**Ile/val(AG)(20)**	**val/val(GG)(1)**	P value
East Indians	**Cases**	19	12	0	.888
	Controls	15	8	1	

7c

		Ile/ile(AA) (13)	Ile/val(AG)(8)	val/val(GG) (3)	P value
South Indians(24)	**Cases**	6	5	0	.223
	Controls	7	3	3	

A subset analysis of the genotype frequencies and their association with TCC bladder was performed among the patients stratified based on the geographic origin i.e. from North India, East India or from South India, as shown in the table 7

Although we observed that majority of our patients were from North-East part of India, there was no significant association with any of the genotypes in various regions with TCC bladder.

Table 8

Subset analysis of the genotype frequencies- For Smoking

Subset analysis of the genotype frequencies- For Smoking					
		Genotype frequency			
		Ile/ile (AA)	**Ile/val(AG)**	**Val/val (GG)**	**p-value**
Smokers	Subjects	n-30	n-13	n-1	0.38
	Cases	22	10	0	
	Controls	8	3	1	

We did a subset analysis to look for genotype frequency among smokers as shown in Table 8. Majority (73.3%) of the smokers with TCC had the wild type allele, a few (31.2%) had ile/val heterozygous genotype, and none of them had homozygous polymorphic valine allele. Even among the control group, majority (66.6%) of the smokers had TCC with wild type homozygous allele and 25% had ile/val heterozygous allele and 0.08 % had homozygous polymorphic allele.

But there was no statistically significant difference among the cases and controls, suggesting that the Polymorphic GSTP1 does not add to the susceptibility of TCC even among smokers.

Summary of results:

Both our groups, the cases as well as controls were well matched for various demographic variables and there was no confounding factor seen. Most of our patients were from the eastern part of India and South India. Majority of the patients were males, probably due to the fact that in India males are more exposed to smoking and also they have higher chances of occupational exposure. Though majority of our patients were aware of the fact that smoking predisposes to cancer, the incidence of smoking was quite high in both the groups. High grade, non muscle invasive tumours were seen more frequently, may be due to higher numbers of smokers in our study group.

The GSTP1 genotype in our study group was in concordance with the Hardy Weinberg equilibrium. We noticed that there was no association of TCC bladder and heterozygous or homozygous polymorphic valine allele in our cohort studied. The subset analysis of the population dividing them into 3 different regions of India as well as according to the smoking habits also did not show any statistically significant association with the GSTP1 polymorphism.

Discussion

GSTP1 plays an important role in protecting cells from the cytotoxic and carcinogenic agents. It forms the thioester bond between the sulphur atom of GSH and the substrate and then catalyzes the conjugation of reduced glutathione (GSH) with the compounds that contain an electrophilic. It also detoxifies the organic hyper-oxides by the reduction reaction.(64)

Altered GSTP1 activity and expression have been reported in many tumors and it is largely due to GSTP1 DNA hypermethylation at the CpG island in the promoter-5' region.(49) The GSTP1 313 A/G polymorphism at the nucleotide level leads to an amino acid variation of isoleucine/valine at codon 105 in the protein. The valine amino acid results in decreased enzyme activity and greater propensity to development of carcinoma.

Polymorphism at the GSTP1 locus is of particular importance since this gene is universally present in many cell types including types including lung (Ryberg et al., 1997), (65) colon (Welfare et al., 1999)(66), and breast cancer (Helzlsouer et al., 1999) (67)

Since this protein is often over-expressed in tumour cells, it can make them resistant to anti cancer treatment.

Role of genetic polymorphism in bladder cancer is being evaluated in various studies done at different centers with heterogeneous ethnic background. Thus, we conducted a case-control polymorphic study to assess the role of Glutathione S transferase GSTP1 polymorphism A/G in urothelial cancers of the bladder in our patient cohort.

The frequencies of the A/A, A/G, and G/G genotypes in our case i.e. TCC group were 63.4%, 34.6% and 1.9% while the frequency in control group were 67.4%, 23.9%, and 8.7%, respectively. The genotypes were in Hardy Weinberg equilibrium among both the cases and the controls.

Our demographic variables were similar to other studies done concerning TCC bladder and GSTP1 polymorphism. The genotype frequency of our control population was similar to what was seen by other Indian studies by Vettriselvi et al., 2006(68) and Pandith et al(63) i.e. AA 67.4%, AG 23.9%, GG 8.7% and AA 58.4%, AG 38.4%, GG 3.1% and AA 76%, AG 22% and GG 2%for ile/ile (AA), ile/val (AG) and val/val (GG), respectively.

We observed no association of GSTP1 A/G or G/G polymorphism with Urothelial cancers of the bladder among both the groups in our study (p- .202). However it was interesting to note that the frequency of heterozygous genotype was seen more frequent among cases. This heterozygous state can lead to in decreased enzyme activity and greater propensity to development of carcinoma.

Similar lack of association was also seen in another study done on Kashmiri population India by Pandith et al in 2013(63) **Our study was also in accordance to a German Study by Steinhoff et al** (62) **and a Japanese study by Katoh et al** (61) **(Table -9)** who observed no association for GSTP1 A/G or G/G genotype with susceptibility to bladder cancer.

Table 9: Studies showing No association between GSTP1 polymorphism and TCC bladder

	Present study 2014		Pandith et al 2013		Katoh et al 1999		Steinhoff et al 2000	
Genotype	**Cases% (n-52)**	**Controls% (n-46)**	**Cases% (n-180)**	**Controls% (n-210)**	**Cases% (n-106)**	**Controls% (n-122)**	**Cases% (n-135)**	**Controls% (n-127)**
ile/ile (AA)	**63.4**	67.4	**71.6**	76	**70.8**	76.2	**50**	55
Ile/val(AG)	**34.6**	23.9	**25.0**	22	**24.5**	19.7	**44**	36
Val/val(GG)	**1.9**	8.7	**3.4**	2.0	**4.7**	4.3	**7**	9

Table 10: Studies showing a Positive association of GSTP1 polymorphism and TCC bladder

	Safarinejad et al 2011		Srivastava et al 2005		Cao et al 2005		Harries et al 1997	
Genotype	**Cases% (n-166)**	**Controls% (n-332)**	**Cases% (n-106)**	**Controls% (n-370)**	**Cases% (n-145)**	**Controls% (n-170)**	**Cases% (n-71)**	**Controls% (n-155)**
ile/ile(AA)	**32.5**	51.8	**31.1**	51.6	**53.1**	54.7	**35.2**	51
Ile/val(AG)	**53.0**	45.8	**54.7%**	44.9	**45.5**	38.8	**45.1**	42.5
Val/val(GG)	**14.5**	2.4	**14.2%**	3.5	**1.4**	6.5	**19.7**	6.5

Our study was in contrary to many other studies as shown in the **table 10**. Srivastava et al in 2005 (58), Safarinejad et al in 2011(34), Cao et al in 2005(69) and Harries et al in 1997 (70) showed a significant association of the polymorphic valine allele with the increased risk for the urothelial cancer of the bladder. All these studies had much higher number of cases as well as controls, as compared to our study.

Smoking is a well known risk factor for TCC of the bladder(69). Smokers in our study had a higher grade of cancer (71). We performed a subset analysis comparing all the smokers with TCC bladder and the smokers seen in the control group. Majority of the smokers with TCC had the wild type allele, a few had ile/val heterozygous genotype, none of them had homozygous polymorphic valine allele **Table- 8**, We noticed no statistically significant difference among the cases and controls, suggesting that the Polymorphic GSTP1 does not add to the susceptibility of TCC even among smokers.

We also attempted to correlate clinical stage and/or pathological grade with GSTs genotypes for the risk of bladder cancer, but no association was observed. Our findings concur with previous studies that also showed non-significant association of GSTP1 genotypes with grade/or stage of the disease

Our hospital caters to a wide variety of populations from all parts of India. We noticed that the cases seen at our centre were derived from predominantly East India and the South India, as shown in the **table 7**. When we performed the subset analysis of the cases according to the regional variations, we found no association of GSTP1 polymorphism with increased or decreased risk of TCC of the bladder.

It is believed that Indian population is genetically heterogeneous, consisting of continuum of the genetic spectrum bridging Central European (CEU) and Chinese (CHB), the two distinct

HapMap populations (72). The north Indians, the North east and the South Indians are believed to have different genetic lineage, though significant admixture has happened over several thousand years. This heterogeneity can explain variability in the genotype frequencies among different subpopulations in India.

The lack of association noticed in our noticed can be attributed to one of the following.

1. Small sample size
2. Complex interaction between polymorphisms of other glutathione enzymes [possibilities of epistasis] e.g. GSTM1 and GSTT1 genotypes as observed by Safarinejad et al(34) and Srivastava et al(58) with GSTP1

This needs further exploration by studying a larger sample size as well as looking in to the polymorphisms of other GST enzymes. The elucidation of complex interactions among different enzymes of glutathione enzymes in the xenobiotic pathways can be attempted by performing epitasis analysis once all the polymorphism frequencies are studied.

Conclusion

Although lack of association between GSTP1313 G/G polymorphism and UC of bladder was seen in our study, it might be due to a small sample size or the effect of complex interaction between polymorphisms of other glutathione enzymes. It is also possible that the polymorphism is not associated with UC of bladder in our population. For further confirmation, large scale population-based studies are required.

Bibliography

References

1. Kurkure A. Cancer incidence and patterns in urban Maharashtra. Consolidated report of the population based cancer registries [Internet]. 2001. Available from: http://www.icmr.nic.in/ncrp/report_pop_2001-04/Initial%20Pages.pdf

2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69–90.

3. Parkin DM. The global burden of urinary bladder cancer. Scand J Urol Nephrol. 2008;42(s218):12–20.

4. Vercelli M, Quaglia A, Parodi S, Crosignani P. Cancer prevalence in the elderly. ITAPREVAL Working Group. Tumori. 1999 Oct;85(5):391–9.

5. Grignon DJ, Ro JY, Ayala AG, Johnson DE, Ordóñez NG. Primary adenocarcinoma of the urinary bladder. A clinicopathologic analysis of 72 cases. Cancer. 1991 Apr 15;67(8):2165–72.

6. Jenney M, Oberlin O, Audry G, Stevens MCG, Rey A, Merks JHM, et al. Conservative approach in localised rhabdomyosarcoma of the bladder and prostate: results from International Society of Paediatric Oncology (SIOP) studies: malignant mesenchymal tumour (MMT) 84, 89 and 95. Pediatr Blood Cancer. 2014 Feb;61(2):217–22.

7. Lerner SP, Schoenberg MP, Sternberg CN. Textbook of bladder cancer. Abingdon, Oxon, UK; Boca Raton, FL: Taylor & Francis; 2006.

8. UK CR. Types of bladder cancer [Internet]. 2013 [cited 2014 Mar 23]. Available from: http://www.cancerresearchuk.org/cancer-help/type/bladder-cancer/about/types-of-bladder-cancer

9. Holmäng S, Andius P, Hedelin H, Wester K, Busch C, Johansson SL. Stage progression in Ta papillary urothelial tumors: relationship to grade, immunohistochemical expression of tumor markers, mitotic frequency and DNA ploidy. J Urol. 2001 Apr;165(4):1124–1128; discussion 1128–1130.

10. Bostwick DG. Natural history of early bladder cancer. J Cell Biochem Suppl. 1992;16I:31–8.

11. Tilki D, Reich O, Svatek RS, Karakiewicz PI, Kassouf W, Novara G, et al. Characteristics and outcomes of patients with clinical carcinoma in situ only treated with radical cystectomy: an international study of 243 patients. J Urol. 2010 May;183(5):1757–63.

12. Farrow GM, Utz DC, Rife CC, Greene LF. Clinical observations on sixty-nine cases of in situ carcinoma of the urinary bladder. Cancer Res. 1977 Aug;37(8 Pt 2):2794–8.

13. Peyromaure M, Zerbib M. T1G3 transitional cell carcinoma of the bladder: recurrence, progression and survival. BJU Int. 2004 Jan;93(1):60–3.

14. Soloway MS, Sofer M, Vaidya A. Contemporary management of stage T1 transitional cell carcinoma of the bladder. J Urol. 2002 Apr;167(4):1573–83.

15. Stein JP, Lieskovsky G, Cote R, Groshen S, Feng AC, Boyd S, et al. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. J Clin Oncol Off J Am Soc Clin Oncol. 2001 Feb 1;19(3):666–75.

16. Fassan M, Trabulsi EJ, Gomella LG, Baffa R. Targeted therapies in the management of metastatic bladder cancer. Biol Targets Ther. 2007 Dec;1(4):393–406.

17. Zhang Z-L, Xiong Y-H, Li Y-H, Hou G-L, Liu Z-W, Han H, et al. Reassessment of the predictive role of perivesical fat invasion in invasive bladder cancer prognosis in 151 Chinese patients. Chin Med J (Engl). 2011 Sep;124(18):2915–9.

18. Bellmunt J, de Wit R, Albiol S, Tabernero J, Albanell J, Baselga J. New drugs and new approaches in metastatic bladder cancer. Crit Rev Oncol Hematol. 2003 Aug;47(2):195–206.

19. Simon R, Eltze E, Schäfer KL, Bürger H, Semjonow A, Hertle L, et al. Cytogenetic analysis of multifocal bladder cancer supports a monoclonal origin and intraepithelial spread of tumor cells. Cancer Res. 2001 Jan 1;61(1):355–62.

20. Grotenhuis AJ, Dudek AM, Verhaegh GW, Witjes JA, Aben KK, van der Marel SL, et al. Prognostic relevance of urinary bladder cancer susceptibility Loci. PloS One. 2014;9(2):e89164.

21. Brennan P, Bogillot O, Cordier S, Greiser E, Schill W, Vineis P, et al. Cigarette smoking and bladder cancer in men: a pooled analysis of 11 case-control studies. Int J Cancer J Int Cancer. 2000 Apr 15;86(2):289–94.

22. Freedman ND, Silverman DT, Hollenbeck AR, Schatzkin A, Abnet CC. Association between smoking and risk of bladder cancer among men and women. JAMA J Am Med Assoc. 2011 Aug 17;306(7):737–45.

23. Mohseni MG, Zand S, Aghamir SMK. Effect of smoking on prognostic factors of transitional cell carcinoma of the bladder. Urol J. 2004;1(4):250–2.

24. Boffetta P, Dosemeci M, Gridley G, Bath H, Moradi T, Silverman D. Occupational exposure to diesel engine emissions and risk of cancer in Swedish men and women. Cancer Causes Control CCC. 2001 May;12(4):365–74.

25. Badawi H, Ahmed H, Ismail A, Diab M, Moubarak M, Badawy A, et al. Role of human papillomavirus types 16, 18, and 52 in recurrent cystitis and urinary bladder cancer among Egyptian patients. Medscape J Med. 2008;10(10):232.

26. Kalisvaart JF, Katsumi HK, Ronningen LD, Hovey RM. Bladder cancer in spinal cord injury patients. Spinal Cord. 2010 Mar;48(3):257–61.

27. Aben KKH, Witjes JA, Schoenberg MP, Hulsbergen-van de Kaa C, Verbeek ALM, Kiemeney LALM. Familial aggregation of urothelial cell carcinoma. Int J Cancer J Int Cancer. 2002 Mar 10;98(2):274–8.

28. Goldgar DE, Easton DF, Cannon-Albright LA, Skolnick MH. Systematic population-based assessment of cancer risk in first-degree relatives of cancer probands. J Natl Cancer Inst. 1994 Nov 2;86(21):1600–8.

29. Lynch HT, Walzak MP, Fried R, Domina AH, Lynch JF. Familial factors in bladder carcinoma. J Urol. 1979 Oct;122(4):458–61.

30. Kiemeney LA, Sulem P, Besenbacher S, Vermeulen SH, Sigurdsson A, Thorleifsson G, et al. A sequence variant at 4p16.3 confers susceptibility to urinary bladder cancer. Nat Genet. 2010 May;42(5):415–9.

31. Kiemeney LA, Thorlacius S, Sulem P, Geller F, Aben KKH, Stacey SN, et al. Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. Nat Genet. 2008 Nov;40(11):1307–12.

32. UK CR. Smoking and cancer: What's in a cigarette? [Internet]. 2012 [cited 2014 Mar 23]. Available from: http://www.cancerresearchuk.org/cancer-info/healthyliving/smokingandtobacco/whatsinacigarette/smoking-and-cancer-whats-in-a-cigarette

33. Cohen SM, Shirai T, Steineck G. Epidemiology and etiology of premalignant and malignant urothelial changes. Scand J Urol Nephrol Suppl. 2000;(205):105–15.

34. Safarinejad MR, Safarinejad S, Shafiei N, Safarinejad S. Association of genetic polymorphism of glutathione S-transferase (GSTM1, GSTT1, GSTP1) with bladder cancer susceptibility. Urol Oncol. 2013 Oct;31(7):1193–203.

35. Kaderlik KR, Kadlubar FF. Metabolic polymorphisms and carcinogen-DNA adduct formation in human populations. Pharmacogenetics. 1995;5 Spec No:S108–117.

36. Bell DA, Taylor JA, Paulson DF, Robertson CN, Mohler JL, Lucier GW. Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen-metabolism gene glutathione S-transferase M1 (GSTM1) that increases susceptibility to bladder cancer. J Natl Cancer Inst. 1993 Jul 21;85(14):1159–64.

37. Savic-Radojevic A, Mimic-Oka J, Pljesa-Ercegovac M, Opacic M, Dragicevic D, Kravic T, et al. Glutathione S-transferase-P1 expression correlates with increased antioxidant capacity in transitional cell carcinoma of the urinary bladder. Eur Urol. 2007 Aug;52(2):470–7.

38. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69–90.

39. Cordon-Cardo C, Cote RJ, Sauter G. Genetic and molecular markers of urothelial premalignancy and malignancy. Scand J Urol Nephrol Suppl. 2000;(205):82–93.

40. Hartmann A, Schlake G, Zaak D, Hungerhuber E, Hofstetter A, Hofstaedter F, et al. Occurrence of chromosome 9 and p53 alterations in multifocal dysplasia and carcinoma in situ of human urinary bladder. Cancer Res. 2002 Feb 1;62(3):809–18.

41. Malats N, Bustos A, Nascimento CM, Fernandez F, Rivas M, Puente D, et al. P53 as a prognostic marker for bladder cancer: a meta-analysis and review. Lancet Oncol. 2005 Sep;6(9):678–86.

42. Baffa R, Letko J, McClung C, LeNoir J, Vecchione A, Gomella LG. Molecular genetics of bladder cancer: targets for diagnosis and therapy. J Exp Clin Cancer Res CR. 2006 Jun;25(2):145–60.

43. Esteller M. Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes. Eur J Cancer Oxf Engl 1990. 2000 Dec;36(18):2294–300.

44. Gene [Internet]. Wikipedia, the free encyclopedia. 2014 [cited 2014 Mar 23]. Available from: http://en.wikipedia.org/w/index.php?title=Gene&oldid=600308119

45. Hedrick PW. Genetics of populations. Sudbury, Mass.: Jones and Bartlett Publishers; 2011.

46. Morrow CS, Cowan KH, Goldsmith ME. Structure of the human genomic glutathione S-transferase-pi gene. Gene. 1989 Jan 30;75(1):3–11.

47. Salinas AE, Wong MG. Glutathione S-transferases--a review. Curr Med Chem. 1999 Apr;6(4):279–309.

48. Allan JM, Wild CP, Rollinson S, Willett EV, Moorman AV, Dovey GJ, et al. Polymorphism in glutathione S-transferase P1 is associated with susceptibility to chemotherapy-induced leukemia. Proc Natl Acad Sci U S A. 2001 Sep 25;98(20):11592–7.

49. Cowell IG, Dixon KH, Pemble SE, Ketterer B, Taylor JB. The structure of the human glutathione S-transferase pi gene. Biochem J. 1988 Oct 1;255(1):79–83.

50. Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol. 1995;30(6):445–600.

51. Sheehan D, Meade G, Foley VM, Dowd CA. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem J. 2001 Nov 15;360(Pt 1):1–16.

52. Ioannides C. Enzyme systems that metabolise drugs and other xenobiotics [Internet]. Chichester; New York: J. Wiley; 2002 [cited 2014 Mar 22]. Available from: http://search.ebscohost.com/login.aspx?direct=true&scope=site&db=nlebk&db=nlabk&AN=78982

53. Spiteri MA, Bianco A, Strange RC, Fryer AA. Polymorphisms at the glutathione S-transferase, GSTP1 locus: a novel mechanism for susceptibility and development of atopic airway inflammation. Allergy. 2000;55 Suppl 61:15–20.

54. Hayes JD, Strange RC. Glutathione S-transferase polymorphisms and their biological consequences. Pharmacology. 2000 Sep;61(3):154–66.

55. Henrique R, Jerónimo C. Molecular detection of prostate cancer: a role for GSTP1 hypermethylation. Eur Urol. 2004 Nov;46(5):660–669; discussion 669.

56. Liu Y-J, Huang P-L, Chang Y-F, Chen Y-H, Chiou Y-H, Xu Z-L, et al. GSTP1 genetic polymorphism is associated with a higher risk of DNA damage in pesticide-exposed fruit growers. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 2006 Apr;15(4):659–66.

57. Törüner GA, Akyerli C, Uçar A, Aki T, Atsu N, Ozen H, et al. Polymorphisms of glutathione S-transferase genes (GSTM1, GSTP1 and GSTT1) and bladder cancer susceptibility in the Turkish population. Arch Toxicol. 2001 Oct;75(8):459–64.

58. Srivastava DSL, Mandhani A, Mittal B, Mittal RD. Genetic polymorphism of glutathione S-transferase genes (GSTM1, GSTT1 and GSTP1) and susceptibility to prostate cancer in Northern India. BJU Int. 2005 Jan;95(1):170–3.

59. Pljesa-Ercegovac M, Savic-Radojevic A, Dragicevic D, Mimic-Oka J, Matic M, Sasic T, et al. Enhanced GSTP1 expression in transitional cell carcinoma of urinary bladder is associated with altered apoptotic pathways. Urol Oncol. 2011 Feb;29(1):70–7.

60. Goerlitz D, El Daly M, Abdel-Hamid M, Saleh DA, Goldman L, El Kafrawy S, et al. GSTM1, GSTT1 null variants, and GPX1 single nucleotide polymorphism are not associated with bladder cancer

risk in Egypt. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 2011 Jul;20(7):1552–4.

61. Katoh T, Kaneko S, Takasawa S, Nagata N, Inatomi H, Ikemura K, et al. Human glutathione S-transferase P1 polymorphism and susceptibility to smoking related epithelial cancer; oral, lung, gastric, colorectal and urothelial cancer. Pharmacogenetics. 1999 Apr;9(2):165–9.

62. Steinhoff C, Franke KH, Golka K, Thier R, Römer HC, Rötzel C, et al. Glutathione transferase isozyme genotypes in patients with prostate and bladder carcinoma. Arch Toxicol. 2000 Nov;74(9):521–6.

63. Pandith AA, Lateef A, Shahnawaz S, Hussain A, Malla TM, Azad N, et al. GSTP1 gene Ile105Val polymorphism causes an elevated risk for bladder carcinogenesis in smokers. Asian Pac J Cancer Prev APJCP. 2013;14(11):6375–8.

64. Cho SG, Lee YH, Park HS, Ryoo K, Kang KW, Park J, et al. Glutathione S-transferase mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1. J Biol Chem. 2001 Apr 20;276(16):12749–55.

65. Ryberg D, Skaug V, Hewer A, Phillips DH, Harries LW, Wolf CR, et al. Genotypes of glutathione transferase M1 and P1 and their significance for lung DNA adduct levels and cancer risk. Carcinogenesis. 1997 Jul;18(7):1285–9.

66. Welfare M, Monesola Adeokun A, Bassendine MF, Daly AK. Polymorphisms in GSTP1, GSTM1, and GSTT1 and susceptibility to colorectal cancer. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 1999 Apr;8(4 Pt 1):289–92.

67. Helzlsouer KJ, Selmin O, Huang HY, Strickland PT, Hoffman S, Alberg AJ, et al. Association between glutathione S-transferase M1, P1, and T1 genetic polymorphisms and development of breast cancer. J Natl Cancer Inst. 1998 Apr 1;90(7):512–8.

68. V V, K V, Paul SF, P V. Genetic variation of GSTM1, GSTT1 and GSTP1 genes in a South Indian population. Asian Pac J Cancer Prev APJCP. 2006 Jun;7(2):325–8.

69. Cao W, Cai L, Rao J-Y, Pantuck A, Lu M-L, Dalbagni G, et al. Tobacco smoking, GSTP1 polymorphism, and bladder carcinoma. Cancer. 2005 Dec 1;104(11):2400–8.

70. Harries LW, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. Carcinogenesis. 1997 Apr;18(4):641–4.

71. Lorente JA, Bielsa O, Rijo E, Cortadellas R, Nohales G, Francés A, et al. Clinical-pathological differences and smoking habit depending on gender in a cohort of patients with transitional cell carcinoma of the bladder: retrospective study. Arch Esp Urol. 2011 Jun;64(5):427–33.

72. Reich D, Thangaraj K, Patterson N, Price AL, Singh L. Reconstructing Indian population history. Nature. 2009 Sep 24;461(7263):489–94.

ANNEXURE

Patient data

Department of Medical Genetics

Name	
CMCH No.	
Date of birth	
Date of sample	
DNA number	Pedigree no.(if applicable)
Name and address of the submitting person	
Diagnostic criteria	1.
	2.
Diagnosis	
Purpose of investigation	Diagnostic
	Research

Department of Urology

Name

Age/sex

HNUM

Residence: North Indian, North- East Indian or South Indian

Education level

Smoker

No of years smoking

No of cigarettes smoking per day

Passive smoking

Awareness that smoking causes cancer

History of cancers in the family

Occupational exposure

Months/ years of occupational exposure

Other risk factors present if any

Reasons for cystoscopy in control group

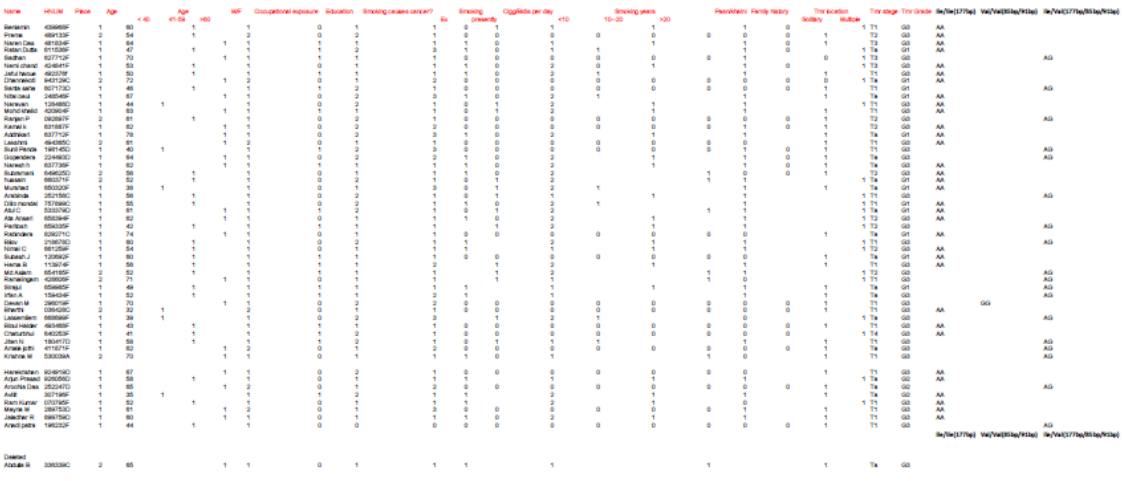
Cystoscopy: Tumours

1. Number
2. Site
3. Papillary or sessile

Histology: Non-muscle invasive disease/ Muscle invasive disease

Grade of the tumour-: Low/High

Genotype: Isoleucine/isoleucine (AA), isoleucine/valine (AG) or valine/valine (GG)



Name	HHUM	Place	Age	Age <40	Age 41-50	Age >50	WP	Occupational exposure	Smoking/cessation cancer?	Education	Rx	Smoking (pack/y)	Cpg/Bidi	Cpg/Bidi <10	Smoking years 10-20	Smoking years >20	Plan/Visit indication for biopsy	Ile/Ile(377bp)	Val/Val(439bp/910bp)	Ile/Val(377bp/939bp/910bp)
Padma K	484277F		1	36	1			2		0		3	1	0	0	0	0	0 Wanting for TR	AA		
Keerthi	842902F		2	57		1		1		1		1	1	0	0	0	0	1 Wanting for Wore da			AG
Archana A	483775F		1	28	1			2		0		1	2	0	0	0	0	0 Biopsy for poor Thin			AG
Shyam R	852375F		1	51		1		1		0		1	1	0	0	0	0	0 Biopsy for uterine calcification	AA		
Mohammed	845175F		1	44	1			1		0		3	1	0	0	0	0	1 Wanting for Wore da			AG
Surender R	283042F		1	55		1		1		0		1	2	1	1	1		1 Wisture-urethra			AG
Sunitha	815208F		1	56		1		1		0		1	1	0	0	0	0	1 Wore da	AA		
Ravindra G	874502D		1	24	1			1		0		1	0	0	0	0	0	0 Wisture-urethra	AA		
Rabeeram	630637F		2	65			1	1		0		1	1	1	1	1		BPH	AA		
Rekha	222503F		2	62		1		1		0		1	1	0	0	0	0	0 Wore da	AA		
Rani	398294F		1	57		1		1		0		1	0	1	1	1		BPH	AA		
Ranjib	457308F		1	42		1		1		0		1	0	0	0	0	0	0 Wore da		GG	
Shyam R Ag	864782R		1	53		1		1		0		1	1	0	0	0	0	0 Wisture-urethra	AA		
Reddi K	426522C		2	52		1		1		0		1	1	1	1	1		0 Wore da		GG	
Kayuli	832098F		2	60			1	2		0		2	0	0	0	0	0	0 Renal mass		GG	
Nooren R	338643V		2	75		1		1		0		1	1	0	0	0	0	0 Wisture-urethra	AA		
Ranjeth S	597234D		1	48		1		1		0		1	1	1	1	1		1 Wore da			
Mohiuddin	831268F		1	38	1			1		0		1	1	0	0	0	0	0 Wisture-urethra	AA		
Nimal	832857V		1	41		1		2		0		1	2	0	0	0	0	0 Renal mass	AA		
Rajendran	832794F		2	53		1		1		0		1	1	0	0	0	0	0 Renal mass	AA		
Kadiri G	835626R		1	26	1			1		0		1	1	0	0	0	0	0 Wore da	AA		
Mika R	829877V		1	49		1		1		0		1	1	0	0	0	0	0 uretaric stricture	AA		
Satyajit	829538F		1	33	1			1		0		1	2	0	1	1		0 Wisture-urethra	AA		
Ramanan	330371C		2	28		1		1		0		1	1	0	0	0	0	0 Wore da			AG
Anupam S	848445V		1	27		1		1		0		1	2	0	0	0	0	0 Wore da			AG
Jothu	829725V		2	63			1	1		0		1	1	0	0	0	0	0 Wisture-urethra		GG	
Rotenthan	830791F		1	40		1		1		0		1	2	0	0	0	0	1 Wore da			AG
Anup C	495236F		1	33	1			1		0		1	2	0	0	0	0	1 Wore da			AG
selvakumar	843158F		2	53		1		2		0		1	0	0	0	0	0	0 Wore da	AA		
Priyalal M	843502F		1	48	1			1		0		1	1	0	0	0	0	0 Wore da	AA		
Jayesh	847845V		1	57		1		1		0		1	2	0	0	0	0	1 BPH			AG
Arul Jothi	491583C		2	38	1			1		0		3	1	0	0	0	0	0 Wisture-urethra	AA		
Nanda	848842F		1	41		1		1		0		2	1	0	0	0	0	0 Wisture-urethra	AA		
Dulal.c	845454F		1	67			1	1		1		1	1	0	1	2	1	1 BPH			AG
Mothakarukh	8811425F		1	38	1			1		0		1	1	0	0	0	0	1 Wore da	AA		
Mahaswary	0875496R		2	75			1	1		0		1	2	0	0	0	0	0 BPH			AG
Tapan K	3833451F		1	62		1		1		0		1	1	0	0	0	0	1 Wisture-urethra	AA		
Ali Aja	8854229F		1	62		1		1		0		1	2	1	2	1		1 Wisture-urethra			AG
Abdullah	398736F		2	39		1		1		0		1	2	0	0	0	0	0 Wisture-urethra	AA		
Pratyasa	8847771F		2	71			1	1		1		1	2	0	0	0	0	0 BPH	AA		
Basanti	8478579F		1	58		1		2		0		2	1	0	0	0	0	0 Wisture-urethra			AG
Dilip	487874D		1	52		1		1		0		1	1	0	0	0	0	0 Wisture-urethra	AA		
Purna D	8508227F		1	43	1			1		1		1	2	0	0	0	0	0 Wisture-urethr			AG
Jai Prakash	2727-BD		1	47		1		1		0		1	2	1	2	1		1 Wisture-urethra	AA		
Sadyendra	842452F		1	28	1			1		0		1	2	1	1	1	0	0 Wisture-urethra	AA		
Lalita jena	825457F		1	68		1		2		0		1	1	1	0	0	0	1 Wisture-urethr	AA		
Sunil D	8521183F		1	77			1	1		0		1	1	1	1	1		0 BPH			AG
Ramanad	869084F		1	69			1	1		0		1	2	0	0	0	0	1 BPH			
John	114153		2	75			1	1		0		1	2	0	0	0	0	0 BPH			
Nallasiwan	8702511		1	69			1	1		0			2	0	0	0	0	1 BPH			
Aditya	8853131		1	67			1	1		0			2	0	0	0	0	1 uretaric stricture			
deleted																					
Vivek N	8549780				1			1		0		1	2	0	0	0	0	0 Wisture-urethra			
Bhavish	448853V					1		1		0		1	1	0	0	0	0	0 Wore da			

	CONTROL NAME	HOSPITAL NO	Iie/Iie(177bp)	Val/Val(85bp/91bp)	Iie/Val(177bp/85bp/91bp)
1	Shayam Sundar Agarwal	684769B	AA		
2	Senthil Kumar	460520C		GG	
3	Balaraman	629007F	AA		
4	Soundar Rajan	338447F	AA		
5	Sanjit Kumar Pramanik	457908F		GG	
6	Siddaiah	222526F	AA		
7	Samit Kumar Pinja	398284F	AA		
8	Lotika jana	625401f	AA		
9	Kuppabai	632689F		GG	
10	Rajib Ghosh	625926F	AA		
11	Rajendran	632784F	AA		
12	Sabjajit Chakraborthy	628538F	AA		
13	Saravanan	330311C			AG
14	Tapan kumar goswami	363341F	AA		
15	Mitra Sengupta	628871F	AA		
16	Sonali Basu	629501F	AA		
17	Mohammed Mofizuddin	631998F	AA		
18	Surendra Kumar Sharma	253042F			AG
19	Kesto	642852F			AG
20	Shyam Shankar Mahato	652379F	AA		
21	Prathima Kumari	484071F	AA		
22	Arup Chandra Dhibar	486928F			AG
23	Archana Agarwal	450751F			AG
24	Jyothi	608701F		GG	
25	Anupam Ghosh	648441F			AG
26	Mohammed Motiurahman	645175F			AG
27	Sudarsan Prasad	629781F			AG
28		616936F	AA		
29	Sandeep Ghosh	974532D	AA		
30	Ponippas	658771F	AA		
31	Dilip Roy	467674D	AA		
32	Basanti Biswakarma	647875F			AG
33	Jai Prakash Chamar	202148D	AA		
34	Purnadeka	655897F			AG
35	Satyendra Singh	642492F	AA		
36	Sunil Dhara	662130F			AG
37	Abdullaha Saood	386180F	AA		
38	Vivek nagpal	569668D	NOT CLEARED		
39	S K Ajij	665423F			AG
40	Bharath	448607F	NOT CLEARED		
41	Mohammed Baharuddin	661142F	AA		
42	Selvakumari	643165F	AA		
43	Priya Lal Majumder	643553F	AA		
44	Nandaprasad Adhikari	649942F	AA		
45	Arul Jothi	491508C	AA		
46	Jaydeb Swarnakar	647841F			AG
47	Dulal Chandra Sahu	645454F			AG
48	Munuswamy	057598B			AG
49	Ram gopal agarwal	668064f	AA		
50	Aditya prasad mandal	665310f	AA		
51	Sardeswar Das	670251f	AA		
52	John	4153	AA		

S NO	PATIENT NAME	HOSPITAL NO	Iie/Iie(177bp)	Val/Val(85bp/91bp)	Iie/Val(177bp/85bp/91bp)
1	Benjamin Laksa	439968F	AA		
2	Prema	489133F	AA		
3	Naren Das	481834F	AA		
4	Ratan Datta	611536F	AA		
5	Sadhan Chandra	627712F			AG
6	Nemi Chand Marwari	424841F	AA		
7	Lakshmi	494365C	AA		
8	Nitai Paul	248546F	AA		
9	Kamal Kumar Sharma	631887F	AA		
10	Dhanakoti	943129C	AA		
11	Santa Saha	607173D			AG
12	Addhikari Mayum Iboton	637712F	AA		
13	Sunil Kumar Panda	198145D			AG
14	Jafrul Vaora	492376F	AA		
15	Chatur Bhuj Patra	640253F	AA		
16	Narayan Chandra Gnani	128486D	AA		
17	Mohammed Khali	420904F	AA		
18	Anadi Patra	196232F			AG
19	Gopendra Acharjee	224493D			AG
20	Naresh Hira	637736F	AA		
21	Ranjan Praaksh Ratuaray	092897F			AG
22	Jithan Nanda	180417D			AG
23	Subramani	649625D	AA		
24	Ata Ansri	658394F	AA		
25	Devan K	296019F		GG	
26	Bharathi	036428C	AA		
27	Irfan Ahmad Quasmi	159434F			AG
28	Sirajul Islam	659985F			AG
29	Ramalingam	428606F			AG
30	Mohammed Aslam	654165F			AG
31	Hema Bauri	113974F	AA		
32	Subash Jaiswal	120692F	AA		
33	Nimai Chand Basak	661259F	AA		
34	Bijoy Patra	218678D			AG
35	Rabindra Kumar Deyhore	829271C	AA		
36	Paritosh Ghosh	659335F			AG
37	Amala Jothi	411871F			AG
38	Krishnamurthy	530039A			AG
39	Atul Chandra Maity	533370D	AA		
40	Dilip Kumar Mondal	757899C	AA		
41	Arabinda Biswas	252156C			AG
42	Mushad Ali	650320F	AA		
43	Hussain	660371F	AA		
44	Mayna Manna	289753D	AA		
45	Arjun Prasad Keshari	926056D	AA		
46	Avijit Das	307196F	AA		
47	Bipul Haldar	493468F	AA		
48	Lalsiemliemlien Joute	668699F			AG
49	Harikrishna Patra	924919D	AA		
50	Ramkumat Kesari	070795F	AA		
51	Arockia Doss	252247D			AG
52	Jhaladhar roy	899759D	AA		